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14. ABSTRACT Sentinel lymph nodes from breast cancer patients have B-- cell antibody producing colonies which can be selected, cultured, expanded and immortalized to provide B-- cell lines expressing anti-- cancer antibodies selective for individual patients or breast cancer subtypes. This proposal is to develop the methodology and application to isolate these anti-- cancer antibody producing B-- cells and effectively immortalize them to produce antibody factories. Antibodies derived from these clones will be used to identify antigen distribution in breast cancers, breast cancer cell lines and other human malignancies. The ultimate goal is to obtain panel of antibodies to human breast cancer antigens designated as such by patient-- specific immune biological responses as opposed to reverse genetic or proteomic methodologies currently being explored. The technological challenges within this proposal will be stepwise overcome with a focus on individual tasks provided in the Statement of Work.					
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Introduction

Sentinel lymph nodes from breast cancer patients have B-cell antibody producing colonies which can be selected, cultured, expanded and immortalized to provide B-cell lines expressing anti-cancer antibodies selective for individual patients or breast cancer subtypes. This proposal is to develop the methodology and application to isolate these anti-cancer antibody producing B-cells and effectively immortalize them to produce antibody factories. Antibodies derived from these clones will be used to identify antigen distribution in breast cancers, breast cancer cell lines and other human malignancies. The ultimate goal is to obtain panel of antibodies to human breast cancer antigens designated as such by patient-specific immune biological responses as opposed to reverse genetic or proteomic methodologies currently being explored. The technological challenges within this proposal will be stepwise overcome with a focus on individual tasks provided in the Statement of Work.

This is a final report for the project. A major drawback was the significant delays due to reduced patient enrollment over the period of the grant. We have overcome this drawback by extending the activity under no cost.

Revision of IRB protocol for collection under DOD award mechanism was completed and approved by the Human Research Protection Office (HRPO) Office of Research Protections (ORP) U.S. Army Medical Research & Materiel Command (USAMRMC) Fort Detrick, Maryland. The following is a summary of entire activity on the project.

Body

Task 1: Isolate, immortalize, select and expand B-cell antibody factories from 60 breast cancer patient sentinel lymph nodes. (Figures provided in below)

1a: Enrollment: Current enrollment during the grant has been 45 samples and research and clinical personnel training for collection of live human samples which require alternative protocols to SOPs for operating room and pathology procedures. The collection goal was not met mainly due to loss of our main breast surgeon performing sentinel lymph node mapping for maternity leave during the active cycle of the grant. Despite that we did have alternative means to collect samples that came through clinic using alternative surgeons on the team.

1b: Analysis of sentinel lymph nodes for B-cell activation. Procedure for immunodetection of B-cell activation areas in a tissue print have been effective and are being applied to every case. **Figure 1** demonstrates merged images of fluorescent signal for CD3 (T- and NK-cells) compared to CD23 (B-cell activation marker) on a tissue print of two nodes from a single case. Note that the circled areas are inversely correlated, that is high CD23 B-cell activation and low CD3 levels. **Figure 2** shows distribution patterns in approximately 30% of two lymph nodes from the same patient. Although both nodes demonstrate many areas of follicles only 2-4 follicles are highly reactive as determined by CD23 expression. **Figure 3** demonstrates even higher magnification immunofluorescence of specific signal for CD20 or CD23 combined with nuclear staining with DAPI to confirm cell density and pattern.

B-cell and non-B-cell core excision is performed on the live lymph node. An example of B-cell core excision is shown in **Figure 4**.

Cell culturing and analysis was performed with flow cytometry and cytospin-immunofluorescence. Determination of cell types in the initial cell pool collected using cytospin

and immunofluorescence shows both CD3 T-cells and many CD20 B-cells. Some of the B-cells are positive for CD23 since this sample was from a CD23 positive core (**Figure 5**). However, a pan cytokeratin antibody showed no tumor cells. Additional cells identified by DAPI staining were likely dendritic and reticular cells derived from the node sample. Figure 6 demonstrates the selectivity of the B-cell core over the non-B-cell core tissue cultured for 3 days demonstrating three-fold selectivity for B-cells.

1c: Immortalization using anti-human IgG activated AKATA EBV cell supernatants was unsuccessful in expanding B-cells from pilot experiments. We have since modified the approach to preserve the cored B-cell region using standard cell cryopreservative medias that permit storage and recovery of the B-cells from the selected lymph node sample. From ten cryopreserved cores, we have explanted and produced pools of immortalized B-cells that expand within multiple 96-well dishes, **Figure 6**. These clones were expanded in culture prior to cryostorage.

Task 2: Identify and test antibody target expression in the source cancer, distribution in a large cohort of breast cancers and non-breast tumors using standard immunological methods.

2a-c: Immortalized B-cells that are frozen in 96-wells have been tested for presence of secreted antibodies in the media prior to freezing. As expected at the early time of immortalization and lack of targeted antigen, there was no selection of secreted IgG or IgM isotypes in the clones obtained from up to 5 cores. We are currently expanding these cultures to determine whether long-term expansion in the presence of tumor lysates can convert some to secretion form antibody production as expected.

2d-e. Antibody evaluation – B-cell clones from 5 cases were plated and secreted Ig antibodies evaluated by simple ELISA. Screening of the secreting cell clones were defined as a total of 125 clones. Of these, immunodetection of human breast cancer cells was performed by flow cytometry and 8 demonstrated cell surface targeting, **Figure 7**. Confirmation was performed on these 8 clones and only 2 demonstrated cancer selective staining on cell lines and one with immunohistochemistry on tumor versus normal tissue samples, **Figure 8**.

Task 3: Evaluate the effectiveness and selectivity of purified antibodies to target estrogen receptor alpha positive/negative breast tumors in vivo using real-time non-invasive imaging.

3a: A pilot analysis of fluorescent dye crosslinking to purified murine albumin has been performed and confirmed by gel electrophoresis (data not shown). Methods are in place to be incorporated with patient-derive antibodies.

3b: Antibody targeting in vitro and in vivo. The single clone identified appears to be capable of targeting aggressive triple negative cancers and less so the ER positive types. Using MDA-MB-231 cells and tumors in immunodeficient mice, we were able to demonstrate selective targeting to tumor but not normal tissues with this antibody.

Key Research Accomplishments

- 1) Completion of all administrative, training, education and implementation of fresh sample collection approved by institution and the USAMRMC.
- 2) Detailed methods for throughput analysis of fresh samples are established as standard operating procedures.
- 3) Highly effective storage and recovery of frozen B-cell zone from the sentinel lymph node has been established.
- 4) Cell viability and primary culture conditions have been established.
- 5) Tested current EBV reagent to assure effectiveness for transforming human B-cells.
- 6) Effective ELISA assay to identify antibody secreting clones.
- 7) Tested method to cross-link and purify antibodies with fluorescent dye needed to perform in vivo tumor targeting experiments.
- 8) Identified clones with secreted antibodies.
- 9) Antibody evaluation by flow cytometry and immunofluorescence identified cell surface targeting antibodies.
- 10) A candidate targeting antibody can selectively identify triple negative breast tumors in vivo without excess distribution in normal tissues.

Reportable Outcomes

Manuscripts – Submitted manuscript under review.

Patent Discovery Submission – Submitted Patent Discovery to University for suitability assessment for patent protection.

Poster Presentations – UCONN Health Center Graduate Student Research Day “Identification of breast cancer sentinel lymph node activation”, Charan Devorakonda PhD candidate.

Presentation: Onyx Pharmaceutical, San Francisco Teleconference July 2013, Merrimack Therapeutics, Boston MA., November 2014.

Meetings and Courses Attended - none

Degrees - none

Employment – Kevin Claffey @ 5%, Daniel Kita @ 100%, Kathryn Phoenix @30%.

Conclusions

Despite a delay in effective throughput of patient samples due to unforeseen circumstances, we are on track to continue to pursue the aims and goals of the proposal. We have been prudent in cost expenditures to be able to extend the project and obtain the goals in the original program despite concurrent delays. Throughput assessment of the clones is extremely laborious and focus on cell surface targeting clones was highly effective. A single potentially cancer selective clone has been identified and is under development for IP protection and pre-clinical efficacy if resources can be obtained to continue.

References

Figures

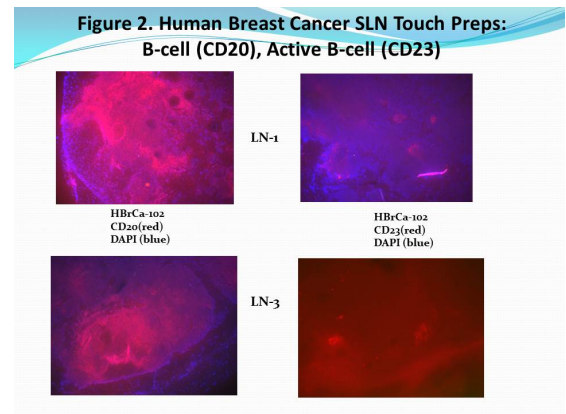
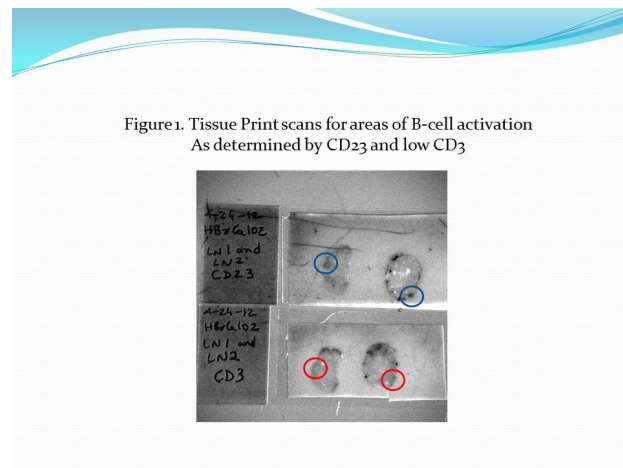


Figure 3. Representative highly activated B-cell germinal center targeted for collection

Localized active germinal center on tissue print of breast cancer sentinel lymph node

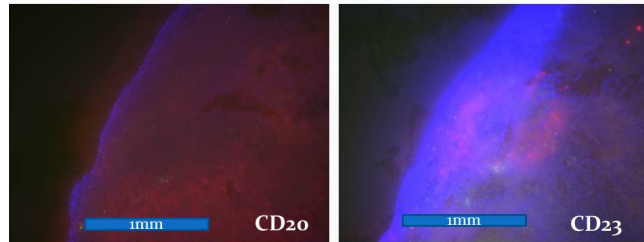
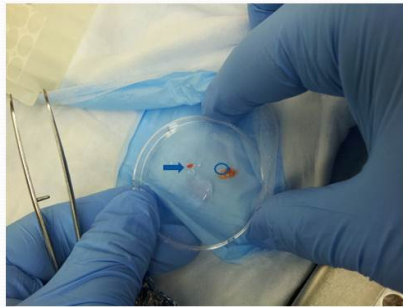


Figure 4. B-cell specific core excision from live lymph node



B-cell core area shown (arrow) derived from selected site of live lymph node (circle)

Figure 5. Cytospin Analysis of B-cell Isolate in a Non-Metastatic Lymph Node

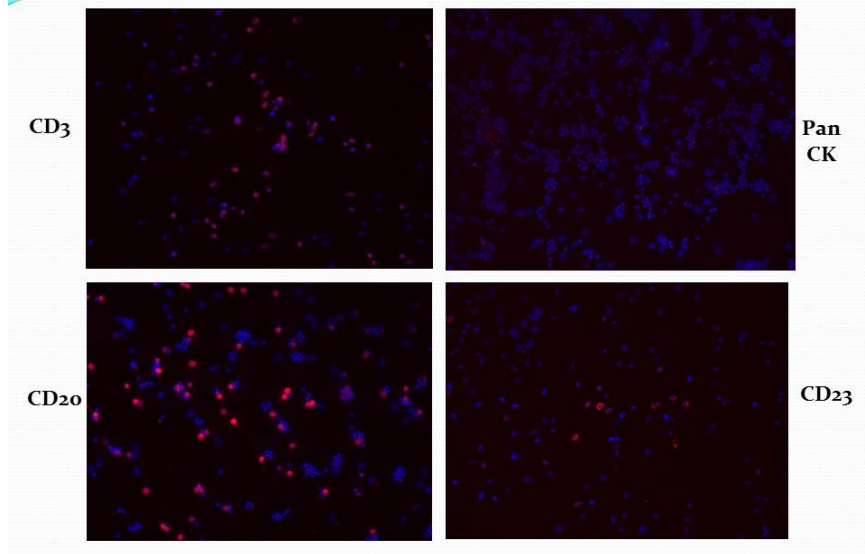


Figure 6. Flow cytometry analysis of the CD3 negative population in 3 day cultures of B-cell core or non-selective T-cell cores.

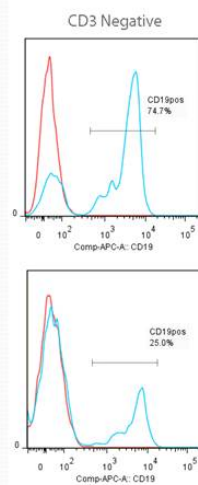


Figure 7. HCAb2 Selective targeting of TNBC cells

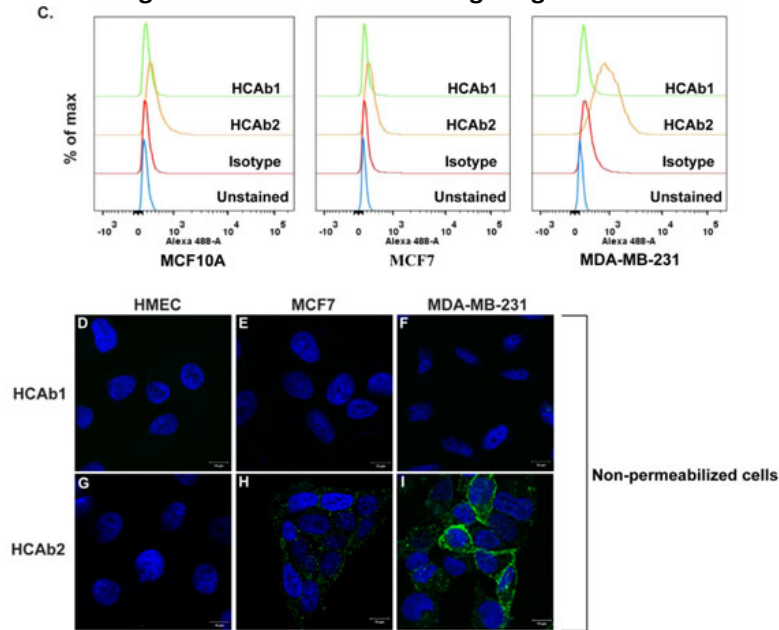
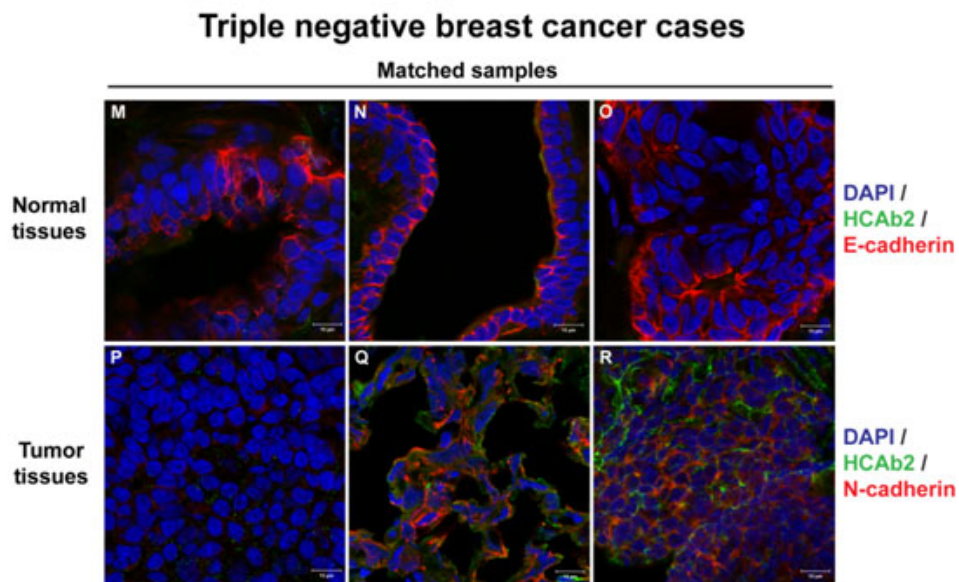


Figure 8. Immunofluorescence of Breast Tumor versus non-tumor in matching patient samples



Appendices

Appendix: N/A